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Characterization of Lactobacillus from Kombucha as Oral Probiotics

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Abstract

In this study, Lactobacillus was isolated from Kombucha beverage, and the Lactobacillus was used as the test object. By detecting the antibacterial effect of its medium supernatant, the strains with antibacterial potential were preliminarily screened. The tolerance to lysozyme and biofilm formation ability, adhesion and biofilm elimination of S. mutans, and the effect on the growth of S. mutans were further evaluated to verify the oral probiotic effect of Lactobacillus screened from kombucha. Finally, 8 strains of Lactobacillus were screened and all of them could tolerate 2mg/mL lysozyme, and all of them had a high ability to inhibit the growth and biofilm formation of S. mutans, with an inhibition rate of 63.84%-76.06%.

Keywords

Lactobacillus Plantarum; Kombucha; Dental Caries; Streptococcus Mutans; Biofilm.

1. Introduction

Dental caries is one of the most common oral diseases in the world, characterized by the demineralization of inorganic matter and dissolution of organic matter, which changes from color to substantive pathological damage with the development of disease course, which is formed by the combined effect of bacteria and plaque, susceptible host and food for a period of time [1]. Among them, bacteria and plaque play an important role in the occurrence of caries, which is the main cause of dental caries [2].

Some studies have shown that the bacteria closely related to caries mainly include Streptococcus, Actinomyces, Lactobacillus, and so on, among which Streptococcus mutans (S. mutans) is an important oral cariogenic bacteria. In the presence of sucrose, S. mutans can synthesize an insoluble extracellular polysaccharide as a supportive framework for the expansion of dental plaque and promote the adhesion of other microorganisms to the enamel surface [3].

In addition to cariogenic bacteria, many studies have shown that some probiotics have the effect of preventing dental caries. Its effects include: probiotic metabolites such as exocytosis inhibit the formation of Streptococcus mutans biofilm; Stimulate host immune response and regulate oral microbiota; Down-regulated or blocked the expression of genes related to the formation of biofilms in S. mutans cells or on the surface of teeth [4].

Among the genera that prevent dental caries, Lactobacillus and Bifidobacterium are the most commonly used probiotic products. Lactobacillus is an important part of the oral microbiota, which is related to the oral health status of individuals. They comprise about 1% of the culturable oral microbiota. Compared with Lactobacillus strains isolated from the oral cavity of individuals with active dental caries, Lactobacillus strains isolated from the oral cavity of individuals without dental caries have the ability to significantly inhibit the growth of S. mutans [5].

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Kombucha is a tea drink fermented from black tea by the symbiotic flora of yeast and bacteria. It is composed of fermented tea broth and bacterial membrane floating on the surface. It tastes sweet and sour and has a unique fermentation flavor. Kombucha has been proved to have cardiovascular and liver protection, anti-inflammatory, antioxidant effects [7,8], and is popular among consumers. In this study, the Lactobacillus extracted from kombucha fermentation was used as the material to investigate its probiotic effect on oral cavity [9-11].

2. Materials and Methods

2.1. Materials and Reagents

Kombucha fermentation broth, indicator bacteria: Escherichia coli, Staphylococcus aureus, Streptococcus mutans, sticky actinomycetes.

MRS Liquid medium (casein digest 10.0 g/L, beef paste powder 10.0 g/L, yeast paste powder 4.0g/L, triammonium citrate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, manganese sulfate 0.05 g/L, dipotassium hydrogen phosphate 2.0 g/L, Glucose 20.0 g/L, Tangu80 1.08 g/L, final pH 5.7±0.2), brain heart extract broth (BHI) liquid medium (peptone 10.0 g/L, sodium chloride 5.0 g/L, disodium hydrogen phosphate 2.5 g/L, glucose 2.0 g/L, Bovine heart extract 500.0ml/L, final pH 7.4±0.2), pancreatic cheese soybean peptone agar (TSA) medium (casein trypsin digestion 15.0 g/L, soybean meal papain digestion 5.0g/L, sodium chloride 5.0 g/L, agar 15.0 g/L, final pH 7.3±0.2), nutrient agar (NA) medium (peptone 10.0 g/L, beef powder 3.0 g/L, sodium chloride 5.0 g/L, agar 15.0 g/L, final pH 7.3±0.1), agar, distilled water, electronic precision balance, Petri dish, conical flask, pH test paper, pipette gun and nozzle.

2.2. Instruments and Equipment

SW-CJ-1F Clean Bench, Shanghai Boxun Industrial Co., LTD. MLS-3750 High pressure steam sterilizer, Tottori, Japan; HPX-9082 MBE, Shanghai Boxun Industrial Co., LTD.; GL-1800 Dry Thermostat, Haimen Qilin Bell Instrument Manufacturing Co., LTD. THZ-D constant temperature oscillator, Changzhou Guohua Electric Co., LTD. Multiskan FC Microplate Reader, Thermo Fisher (Shanghai) Instruments Co., LTD. ZF-90 dark box type ultraviolet transmissive instrument, Shanghai Gucun Electro-Optical Instrument Factory; DYY-7C electrophoresis apparatus, Beijing Liuyi Instrument Factory.

2.3. Isolation of Lactobacillus

Using the coating plate method, 10 μL of kombucha fermentation broth purchased from Liaoning, Anhui and Guangdong provinces was spread on the surface of MRS Medium, and the culture was incubated at 37 ° C for 24h. The colonies with good growth trend were selected, separated and purified repeatedly by plate line separation method, and numbered separately to facilitate later experiments.

2.4. Evaluation of Broad-spectrum Antibacterial Activity of Lactobacillus

After the isolated Lactobacillus were activated with MRS Liquid medium, the numbered Lactobacillus were inoculated into corresponding test tubes, and incubated at 60r/min for 24h in a constant temperature oscillating incubator at 37 ℃. Cell-free fermentation supernatant was prepared using 0.22 μm sterile filter. Using Staphylococcus aureus and Escherichia coli as indicator bacteria, MRS Liquid medium as negative control, gentamicin as positive control, the Oxford cup double plate method was used to screen out the strains with obvious inhibition circle to each indicator bacteria. Each experiment was repeated three times, and the diameter of the inhibition zone was measured by the cross crossing method.

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The supernatant of Lactobacillus was prepared, and the pH value of the supernatant of the fermentation broth of each Lactobacillus was neutralized to 5.5 with 3moL/L NaOH solution. The unneutralized supernatant and the buffer solution of citrate and disodium hydrogen phosphate with pH adjusted to 5.5 were used as the control. 100 μ L samples were added to each well. The plates were incubated at 37 ℃ for 12 hours, and the bacteriostatic circles were measured and recorded to exclude false positives in the bacteriostatic activity test of Lactobacillus fermentation supernatant due to acidic pH.

Different Lactobacillus plantarum fermentation supernatants were incubated at 70 ℃ for 30min, and the supernatant without heat treatment was used as a control. The supernatant was neutralized to pH 5.5 and filtered with 0.22 μ m filter. The double Oxford cup method was used to test the thermal stability of potential antimicrobial compounds in Lactobacillus fermentation broth.

2.5. Screening of Bacteriostatic Oral Pathogenic Bacteria Lactobacillus

The lyophilized bacteria powder of Streptococcus mutans and viscous actinomycetes in lyophilized tubes were dissolved with BHI liquid medium and TSA liquid medium, respectively. The dissolved bacterial suspension was transferred to BHI solid medium and TSA solid medium, respectively, and the inoculated medium was cultured anaerobically in an incubator at 37 ℃.

The selected Lactobacilli were activated with MRS Liquid medium, inoculated in corresponding test tubes, and incubated at 60r/min at 37 ℃ for 24h in a constant temperature oscillating incubator. Cell-free fermentation supernatant was prepared using 0.22 μ m sterile filter. With Actinomycetes viscous and Streptococcus mutans as indicator bacteria and MRS Liquid medium as negative control, the strains with obvious inhibition zones to each indicator bacteria were screened by Oxford cup double-layer plate method. Each experiment was repeated three times, and the diameter of the inhibition zone was measured by the cross crossing method.

2.6. Inhibition of Streptococcus Mutans Biofilm Formation

40 μ L of Streptococcus mutans suspension and 80 μ L of filtered Lactobacillus supernatant were added to the 96-well plate, respectively. The same volume of blank MRS Liquid medium was used as negative control. Each well was tested in triplicate, and the culture was anaerobic at 37 ℃ for 48h. At the end of the culture, the biofilm was washed twice with PBS, fixed with 99% methanol for 15min, dried at room temperature for 10min, and then stood at room temperature to dry the biofilm. Then 100 μ L of 0.1% crystal violet solution was added to each well, and the biofilm was stained for 30min. After the staining, the biofilm was washed twice with PBS again. Then, 100 μ L of 95% ethanol was added to dissolve, and the absorbance value was read by OD620 after shaking on a shaker for 30min.

2.7. Evaluation of the Ability of Automated Polymerization of Lactobacillus

1mL of Lactobacillus suspension to be tested (initial OD620=0.6±0.02) was placed in a 1.5mL microcentrifuge tube and cultured anaerobically at 37 ℃. The absorbance value of the supernatant at 620nm was measured every 1h for a total of 3h. Three parallel experiments were set for each group.

2.8. Determination of Self-biofilm Formation Ability of Lactobacillus sp

The activated Lactobacillus was inoculated in BHI medium containing 3g/L sucrose and incubated anaerobically at 37 ℃ for 18h. The OD620 was adjusted to 0.5±0.02 with blank BHI medium containing 3g/L sucrose, and then 0.2mL of bacterial suspension was added to the 96-well plate. The cultures were incubated at 37℃ for 24h, free bacteria were discarded, and each well was gently washed three times with 0.2mL deionized water. After natural drying, 0.05mL10g/L crystal violet solution was added to each well and stained for 15min at room

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temperature to stain the adhering bacteria. After decanting the staining solution, wash with deionized water for more than 3 times. After drying, 0.2mL ethanol/acetone mixture was added to each well to fully dissolve the crystal violet in the well, and the absorbance was measured at 620nm wavelength with microplate reader. Streptococcus mutans biofilm formation ability under the same conditions as the control group, each experimental group do more than 3 parallel, repeated 3 times.

2.9. Evaluation of Tolerance of Lactobacillus to Lysozyme

The suspension of screened strains and solid MRS Medium were mixed and poured into a plate, and then placed in an Oxford cup after solidification. 100μL of lysozyme solution with different concentrations (0, 0.4, 0.8, 1.2, 1.6, 2.0mg/mL) was added to the Oxford cup, and incubated at 37℃ for 24h. The tolerance of Lactobacillus to lysozyme was determined according to the degree of clear circle.

3. Results and Analysis

3.1. Screening of Lactobacillus with Antibacterial Activity

A total of 32 strains were screened. The bacteria to be tested were blue-purple and rod-shaped, indicating Gram-positive bacteria. The results showed that 32 strains showed different inhibitory effects on Staphylococcus aureus and Escherichia coli, among which the strains numbered 3, 5, 11, 12, 17, 18, 21 and 24 showed significantly stronger inhibitory effects than the other strains and the control group.

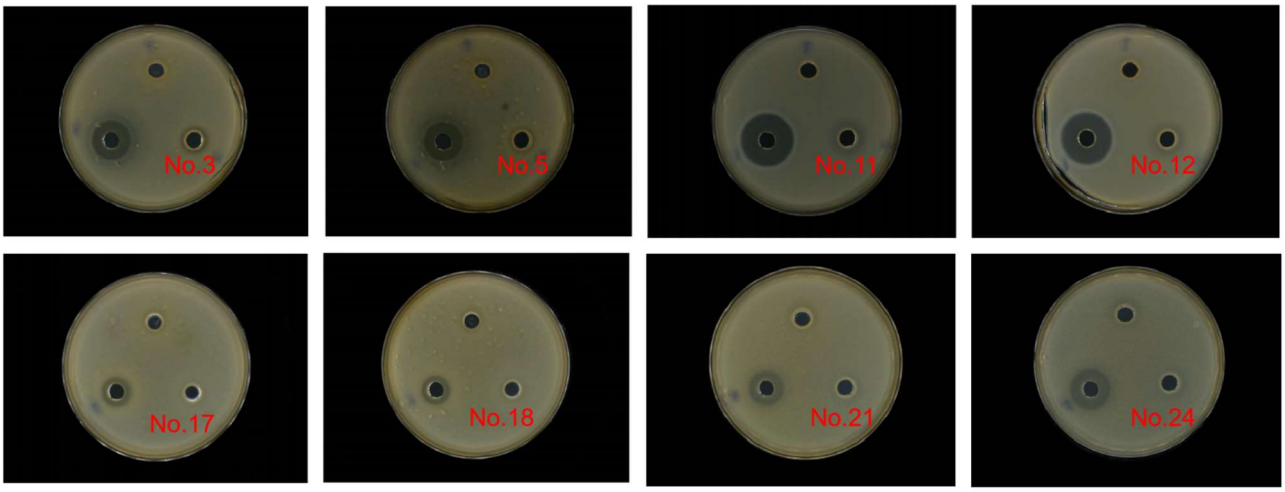

Fig. 1. Typical picture of Lactobacillus fermentation broth antibacterial test

The experimental results show that the antibacterial effect of the 8 strains screened above with broad-spectrum antibacterial effect is weakened or lost compared with the untreated supernatant after acid neutralization and heat treatment, indicating that the antibacterial effect of the supernatant of these strains is related to the acid and antibacterial peptide produced in the process of bacterial metabolism.

3.2. Screening of Lactobacillus with Potential to Inhibit Oral Pathogens

With streptococcus mutans and viscous actinomycetes as indicator bacteria for double plate method of preliminary experiment preliminary validation 3, 5, 11, 12, 17, 18, 21, 24 strains that the bacteriostatic effect of supernatant fluid, the strain in this experiment to some extent bacteriostatic circle, more than inhibition of actinomycetes strains of streptococcus mutans and viscous effect are shown in table 1.

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Table 1. Inhibitory effect of Lactobacillus fermentation broth on Streptococcus mutans and viscous Actinomycetes (Oxford Cup method)

Strain number	Diameter of inhibition zone against Streptococcus mutans culture (mm)	Diameter of inhibition zone against viscous actinomycetes culture (mm)
No.3	12.07	14.31
No.5	12.65	15.26
No.11	10.87	0
No.12	0	0
No.17	11.96	15.38
No.18	12.56	15.89
No.21	0	0
No.24	11.48	0

The bacterial strains were screened and evaluated by measuring their ability to inhibit biofilm formation of cariogenic bacteria. The experimental results showed that: Compared with the blank MRS, the above selected strains had significant elimination effects on the biofilm of S. mutans, and the elimination rates were 63.84%, 75.80%, 73.10%, 75.16%, 75.93%, 76.06% and 70.14%, respectively.

Streptococcus mutans in oral cavity is the main pathogenic bacteria leading to dental caries. The biofilms produced by the above screened strains of Streptococcus mutans have different degrees of elimination effect, which may play a certain role in preventing dental caries.

Oral lysozyme tolerance was evaluated by measuring the selected strains. After culture at 37 ℃ for 24 h, there was no bacteriostasis circle formed around the Oxford cup with lysozyme concentration of 0, 0.4, 0.8, 1.2, 1.6, 2.0 mg/mL, indicating that the selected strains could tolerate lysozyme concentration of 2 mg/mL. It is much higher than the mass concentration of lysozyme in human oral cavity (1-57μg/mL), so these strains have the ability to survive in oral cavity.

The self-copolymerization ability of the selected strains and the co-copolymerization ability of Streptococcus mutans were determined. The self-copolymerization ability of the strains played an important role in the survival of the strains in the oral cavity, which could improve the concentration of probiotics in the oral cavity and make them play a better function. The stronger the autoaggregation ability or copolymerization ability of strains, the more conducive to the colonization, reproduction and survival of strains in the oral cavity, which is an important prerequisite for strains to exert probiotic properties in the oral cavity.

The biofilm formation ability of the selected strains and the ability to inhibit the biofilm formation of cariogenic bacteria were determined and evaluated. As potential oral probiotics, their ability to self-form biofilms is also an important measure. In order to avoid promoting the formation of biofilm in oral cavity, the biofilm formation ability of oral cavity should be weak. The results showed that strains No.3, NO.5, NO.11, No.12, No.17, NO.18, NO.21 AND NO.24 had certain biofilm forming ability, but it was weaker than that of S. mutans.

4. Conclusion

The occurrence and development of oral diseases are closely related to oral flora. Nowadays, probiotics, as a new way to prevent and treat diseases, has been gradually recognized by the public, and has been applied in many fields, including oral care, etc., and many oral care products containing probiotics have appeared in the market. Probiotics can colonize the oral

mucosa and compete for adhesion points with oral pathogenic bacteria. The metabolites of probiotics can also play a beneficial role. A variety of studies have proved the prevention and treatment effect of probiotics on oral diseases, which provides a reference for the development and application of oral probiotics in the field of oral care in the later stage.

Eight strains screened from kombucha fermentation broth were able to tolerate high concentration of lysozyme and survive in oral cavity. It showed certain ability of self-polymerization and co-polymerization with Streptococcus mutans, which was conducive to its stay in the mouth. More importantly, they showed strong elimination effect on Streptococcus mutans biofilm. Therefore, these 8 strains of Lactobacillus have certain potential for oral probiotics application.

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